Author's response to reviews

Title: The expression of microRNA-375 in plasma and tissue is matched in human colorectal cancer

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Author's response to reviews: see over
The expression of microRNA-375 in plasma and tissue is matched in human colorectal cancer (MS: 1750449064129322)

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Authors: Lingling Xu, Minzhe Li, Min Wang, Dong Yan, Guosheng Feng, Guangyu An

Dear editor,

This is a re-submission of MS: 1750449064129322. Thank you for giving us the opportunity to revise the manuscript. Those comments are all valuable and very helpful for revising and improving the manuscript. We have studied the comments carefully and revised the manuscript accordingly. The changes to the manuscript have been reviewed and approved by all authors. In the following pages we have addressed the major and minor queries point-by-point.

We hope that the revised manuscript is now acceptable for publication in BMC cancer and look forward to hearing from you again.

Thank you and best regards.

Yours sincerely,

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Responds to the reviewer’s comments

Reviewer: Joana Carvalho

Major Queries

Q1 Abstract

The authors should better clarify the aim of the study. For instance, they should mention what they mean by significance in CRC. Are they looking for non-invasive diagnostic, prognostic or predictive biomarkers?

"In the screening phase, a set of 42 miRNAs were gained which showed..."With this sentence, it seems that all 42 miRNAs were upregulated or gained at the genomic level..., Please re-phrase.

Response:

According to the reviewer’s suggestion, to obtain a better understanding of the aim of the study, last sentence of the background in the abstract was changed to emphasize the significance of the study. The revised sentence was on page 2, line 46-47. The sentence of "In the screening phase..." was re-phrased to avoid misunderstanding on page 2, line 59-60.

Q2 Background

In general, the introduction is well organized although some references should be added particularly in sentences 95-96,98. The last paragraph of the background section should be more carefully written without detailed information concerning materials and methods. For instance the aims of the study should be clearly written and a brief description of the main findings should be pinpointed.

Response:

On page 4, line 84-85: two references were added to explain the incidence rate and progress of CRC in recent years. The last paragraph of the background section was re-phrased. Some materials and methods were deleted. The aim of the study was to define tissue and plasma miRNAs
signatures, which could potentially serve as diagnostic markers in CRC. The objective and the main findings of the study were stated in the last paragraph of the background.

Q3 Materials and Methods
It would be nice to see the clinicopathological characteristics of all patients and tumours summarized in a table. It would be nice to have more clinical data on table 1 and consider presenting it in this section.

Response:

According to the reviewer’s suggestion, to obtain a better understanding of all the participants enrolled in the study, some clinicopathological characteristics of all patients were added in Table 1.

Results

Q4 Circulating miRNA microarray profiling
The authors have mentioned that 42 miRNAs were differentially expressed when comparing plasma samples of 6 CRC patients with plasma samples of 6 healthy controls. Besides this description, it would be nice to see hierarchical clustering images of the differentially expressed miRNAs. In the legend of table 2, it would be nice to explain what is the meaning of fold change.

Response:

According to the reviewer’s suggestion, we have added Supplementary Fig. S1 which shows hierarchical clustering analysis of the plasma array. The cluster analysis of 42 differential miRNAs was performed by Cluster 3.0 software. Red represents up-regulation and green represents down-regulation. Fold change means $2^{\Delta\Delta CT}$, $\Delta CT = CT_{mean}(miRNA)-CT_{mean}(U6)$, $\Delta\Delta CT=\Delta CT_{CRC}-\Delta CT_{control}$. The explanation of fold change was added in the legend of Table 2.

Validating of selected miRNAs by qRT-PCR

Q5 It would be nice to see the plots representing the expression levels of the 5
miRNAs in the screening and validation series. By comparing the data described in table 2 and table 3 (plasma), only miR-206 was in fact validated and found to be highly expressed in the plasma of CRC patients. Do the authors have any explanation for this?

Response:

According to the reviewer’s suggestion, to obtain a better understanding of the expression levels of 5 miRNAs in the screening and validation series, the plots of 5 miRNAs in the screening phase were added in Supplementary Fig. S2.

We have investigated previous literature about miR-206 in cancer. It was found that miR-206 is a circulating muscle-specific miRNA. The expression of serum miR-206 is significantly higher in rhabdomyosarcoma[1] and in the early stage of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) induced lung carcinogenesis[2]. However, the expression of miR-206 is down-regulated in some tumor tissue sample, such as breast, lung, gastric and colorectal cancer[3-6]. Presently, few reports have been published that concern the significance of circulating miR-206 in CRC. The reason of inconsistent expression pattern of miR-206 in tissue and fluid samples remains largely unknown. These explanations were added in the discussion section on page 11, line 359-366.

Q6 The authors should clarify what was the purpose of using a screening and validation series, since they have not pursue the only miR that was in fact validated.

Response:

We thank the reviewer for this comment. In the present study, the expression levels of tissue or circulating miRNAs have been extensively reported, however, there are few reports addressing the expression of miRNAs in tissue and matched plasma samples. Therefore, the purpose of our study is to assess the expression of miRNAs in tissue and matched plasma samples. In
the screening phase, we used the TaqMan Low Density MiRNA Arrays (TLDAs) to perform miRNA analysis of our discovery cohort. Due to the small sample size (CRC n=6, healthy controls n=6) and the heterogeneity of the tumors, real-time PCR was used to validate the miRNAs.

Heterogeneity is the nature of tumors and may give rise to challenges to all experimental and clinical studies, especially to biomarker development and personalized-medicine. Clinical samples have a great variability. Due to the small sample size and individual differences, the result of validation set is more credible. In the design of our study, we prepared to get a pool of miRNAs to be blood biomarkers for the early detection of CRC. Therefore, 5 miRNAs were chosen for validation. Combined with the results of screening and validation phrase in the plasma and tissue samples, only miR-375 showed consistent correlations between tissue and plasma samples. Moreover, plasma miR-375 has a stronger differentiation power than tissue miR-375 individual or combination with other miRNAs. Therefore, target prediction and function analyses were carried out for miR-375. We have added relative explanations in the result section on page 8, line 233-235.

Q7 Although, the author have mentioned that the expression levels of miR-150, miR-125b and miR126* were not statistically different when comparing plasma of CRC patients and plasma of controls, it would be nice to add these plots to figure 1. In the same line, please add the plot of miR-206 expression in tissues to figure 2. The legend of x-axis of figure 1 is confusing. Please consider instead of "T" writing CRC patients (n=88) and instead of "N", writing healthy controls(n=40).

Response:
According to the reviewer’s suggestion, the plots of expression of miR-150, miR-125b and miR126* in plasma samples were added to figure 1. The plots of expression of miR-206 in tissue samples were added to figure 2. The legend of x-axis of figure1 was revised according to the reviewer’s suggestion.
Q8 It would be nice to analyze the expression level of miR in CRC tissues and matched normal mucosas by using the $2^{\Delta\Delta CT}$ in order to better understand how the expression of each selected miR varies when comparing tissue carcinoma and matched normal mucosa of each patient.

Response:

We thank the reviewer for this comment. In our study, the expression level of miR in CRC tissues and matched normal mucosas were analyzed by $2^{\Delta\Delta CT}$. Because tumors are heterogeneous and clinical samples have a great variability, this method of analysis $2^{\Delta\Delta CT}$ can account for inter-individual variability. There are some results presented by the $2^{\Delta\Delta CT}$ in the clinical samples[7-9]. The results of the analysis are shown in figure 1 and 2.

Discussion

Q9 Concerning the current study, did the expression levels of these miRNAs associate with some of the clinicopathological variables of CRC patients?

Response:

The clinicopathological features of CRC patients in the validation cohort and summary of results in validation phase of the study were shown in Supplementary Table. S1-2. The results reveal that none of the miRNAs either in tissue or plasma samples has significant impact on clinicopathological features. The relative explanations were added in the section of results and discussion on page 8-9, line 254-257 and page 11, line 356-358.

Q10 It was recently identified a panel of miRNAs that could be of potential use in the development of a multi-marker blood test for early detection of CRC (PLOS ONE 2013 May 14;8(5):e62880.doi:10.1371/journal.pone.0062880). The authors should comment on this in comparison with the results obtained in their study.

Response:

This paper published in Plos One is the first one using Taqman microRNA Array for miRNA profiling in CRC detection. The author identified a panel of circulating miRNAs which could be non-invasive biomarkers for CRC detection.
In the screening phase, pooled plasma samples from 50 CRC patients and 50 neoplasma-free controls were used. They used five pools of plasma samples from ten CRC patients each and five pools of plasma samples from ten neoplasma-free controls each. This is different from our study. Besides, this research focuses on the expression of plasma miRNAs in different stages. They grouped the participants into neoplasma-free controls, advanced adenoma patients, early stage of CRC and late stage of CRC. However, our study focuses on the expression of miRNAs in different samples. We want to known whether the circulating miRNAs are reflective of those in the tissue. We compare the expression of miRNAs in plasma and matched tissue samples and find whether the expression of miRNA is consistent in different samples. The relative explanations were added in the discussion section on page 10, line 300-303.

Q11 Did the authors have any possible explanation for the higher expression levels of miR-125b and miR-206 in the plasma of CRC patients in comparison with the corresponding tumor tissue?

Response:

We have investigated previous literature about miR-206 and miR-125b in cancer. miR-206 is a circulating muscle-specific miRNAs. The expression of serum miR-206 is significantly higher in rhabdomyosarcoma[1] and in the early stage of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung carcinogenesis[2]. However, the expression of miR-206 is down-regulated in some tumor tissue sample, such as breast, lung, gastric and colorectal cancer[3-6]. Presently, few reports have been published on the significance of circulating miR-206 in CRC. As such, the reason of inconsistent expression pattern of miR-206 in tissue and fluid samples remains largely unknown.

Circulating miR-125b is a potential biomarker to predict the prognosis and chemoresistance in lung, breast cancer and colorectal cancer[10-13]. The expression level of miR-125b is significantly increased in the cancer patients
and the high level of circulating miR-125 is an independent poor prognostic factor for survival. In colorectal cancer, high expression levels of miR-125b were associated with late stage and poor prognosis. miR-125b is multi-faceted, with the ability to function as a tumor suppressor or an oncogene, depending on the cellular context[14]. There is no report about the expression of miR-125b in plasma and matched tissue samples in CRC. Recently, a study revealed that the expression level of miR-125b in exosomes were significantly lower in patients melanoma compared with disease-free patients with melanoma and healthy controls[15]. Exosomes can provide a suitable material to measure circulating miRNAs in melanoma. Therefore, the expression of miR-125b have not concluded the consistent results so far. We have added relative explanations in the discussion section on page11-12, line 359-377.

Q12 Did the author know which mechanism or mechanisms are underlying miR-150,miR-125b,miR-206,miR-126* as well as miR-375 de-regulation in CRC?

Response:

miRNAs are short non-coding RNAs that regulate messenger RNA(mRNA) or protein levels either by promoting mRNA degradation or by attenuating protein translation. Some mechanisms of miR-150, miR-125b,miR-206,miR-126* and miR-375 have not been clearly understood in CRC.

It has been reported that miR-150 was down-regulated in CRC tissue and was associated with survival and response to adjuvant chemotherapy[16]. But the mechanisms of the dysregulated miR-150 have not been elaborated in CRC, though it has been studied in other cancers. In lung cancer, miR-150 was found to be significantly upregulated in lung cancer clinical specimens and cell lines[17]. miR-150 targets SRCIN1. miR-150 directly recognizes the 3’UTR of SRCIN 1 transcript. The repression of SRCIN1 by miR-150 consequently triggered the activation of the Src/focal adhesion linase(FAK) and Src/Ras/extracellular signal-regulated kinase (ERK) pathway, which eventually
promoted the proliferation of migration of lung cancer cell lines. Similarly, miR-150 was over-expressed in breast cancer cell lines and tissues and promotes human breast cancer growth. The pro-apoptotic purinergic P2X7 receptor was the target of miR-150[18]. miR-150 also is associated with prognosis in other carcinoma, such as pancreatic, esophageal squamous cancers by targeting MUC4, ZEB1[19, 20].

For miR-125b, it has been reported that miR-125b was down-regulated in CRC tissue and was associated with tumor invasion and poor prognosis[21]. The target of miR-125b is Mcl-1, Bcl-w, IL-6R to promote apoptosis. Other study also report that the over expressed miR-125b repressed the endogenous level of P53 protein and focused on the miR-125b/P53 pathway[13]. The results shows that miR-125b is directly involved in cancer progression and is associated with cancer progression and poor prognosis.

To our best knowledge, there are few studies on miR-206 in CRC. A study revealed that miR-206 was down-regulated in CRC tissue samples and was associated with clinical stage, lymph node metastasis and poor survival[6]. But the mechanisms of miR-206 in CRC remains largely unknown. A recent study of miR-206 in melanoma showed that it targeted CDK4, Cyclin C and Cyclin D1 which were cell cycle genes. Therefore, miR-206 induced G1 arrest and acted as a tumor suppressor in melanoma[22].

There are few studies on miR-126* in CRC. miR-126* is the complementary sequence of miR-126. However, the expression of miR-126 has been validated in CRC. It had been revealed that miR-126 was downregulated in CRC tissue samples. miR-126 was downregulated in CRC tissues that expressed high levels of CXCR4. The low miR-126 and high CXCR4 protein expression was associated with distant metastasis, clinical TNM stage and poor survival[23]. miR-126 overexpression inhibit cell proliferation, migration and invasion, and induced cell arrest in the G0/G1 phase of CRC cells. The results revealed that miR-126 function as a tumor suppressor in CRC cells by regulating CXCR4 expression via the AKT and ERK1/2 signaling pathways[24].
It has been revealed that miR-375 was down-regulated in CRC tissues and lines[25]. miR-375 suppressed CRC cell proliferation and colony formation and led to cell cycle arrest. PI3K is the target of miR-375. miR-375 overexpression resulted in inhibition of PI3K/Akt signaling pathway[26]. Another research revealed that miR-375 reduced cell viability through the induction of apoptotic death. YAP1 was the direct target of miR-375. It seemed that miR-375 exerted its pro-apoptotic role through YAP1 and its anti-apoptotic down-stream targeted BIRC5 and BCL2L1[27]. We have added relative explanations in the discussion section on page 10-11, line 307-340.

Q: Minor issues not for publication

Typographical errors:
-phase instead of "phrase" (abstract and results section)
-target instead of "targeted" (materials and methods section)
-verify instead of "verity" (results section)

Response:
We are very sorry for our spelling errors. We have revised the errors in the manuscript.

The references are as follows:


3. Li Y, Hong F, Yu Z: Decreased expression of microRNA-206 in breast cancer and its association with disease characteristics and


Responds to the reviewer’s comments

**Reviewer: Shuhong Luo**

**Major Points**

**Q1** The major weakness of this manuscript is not addressed the molecular mechanism of miR-375. The study of function of miR-375 will benefit the manuscript.

**Response:**
We thank the reviewer for this comment. In our study, the target genes and the function of miRNAs were predicted by an integrated database. The results showed that gene regulated by miR-375 participated in most of the important biological process such as growth or developmental process and function as transcription regulators or molecular transducers which were closely related with the development and progression of cancer. Please see page 9, line 282-291. We are going to study the molecular mechanism of miR-375 in subsequent research. The relative explanations were added in the section of discussion on page 12, line 396-397.

**Q2** There are many reports to identify miR-375, 150, 206, 125b and 126*(see reference 20-31). The novelty of the present study should be discussed.

**Response:**
Although the expression levels of miRNAs have been extensively reported in CRC either by analyzing tissues or plasma samples, there are few reports addressing miRNAs expression in tissue and matched plasma samples of CRC. References mentioned in the manuscript (reference 23-39) report the
expression level of miRNAs in tissue or plasma samples of CRC patients. In this study, tissue and matched plasma samples were collected. The aim of our study is to known whether dysregulated expression of miRNAs in tissue or circulation is consistent. The relative explanations were added in the section of background and discussion on page 5, line 119-125 and page 10, line 302-306.

**Minor comments:**

**Q1** In the validation phase, five miRNAs were chosen for further validation. Why? This should be discussed.

**Response:**
In the screening phase, 42 miRNAs were found differentially expressed. In the condition of Fold change>2.0 and \( p<0.05 \), we obtained a set of 16 miRNAs. Then we investigated the previous literature on these 16 miRNAs. Based on the significance of the difference (fold change, \( p \)-value), previous observations and biological plausibility (according to putative miRNA targets and/or Pubmed hits when particular miRNA is combined with keyword “cancer”), and favorable expression levels (\( C_T<30 \)), we found that these five miRNAs (miR-375, miR-150, miR-125b, miR-206 and miR-126*) were more correlated with CRC. Therefore, we chose these five miRNAs for the validation. The relative explanations were added in the section of methods on page 6, line 167-170.

**Q2** Line 68, Page 3. Authors should add the \( p \) value of correlations between plasma and values after \( r \) value.

**Response:**
We have added the \( p \) value of the correlation.

**Q3** There are numerous databases to predict the miRNAs target gene, why did you choose these four databases? This should be discussed.

**Response:**
To better understand the mechanisms of miRNAs, much progress has been
made in computational target prediction of miRNAs in recent years. More than 10 miRNA target prediction programs have been established, however, the prediction of animal miRNA targets remains a challenging task. Some studies have revealed that the databases of PicTar and Targetscan had a high specificity and was more stability to predict the target genes[1]. Tarbase and miRecords included some target genes which had been validated in the research. Therefore, we chose these four databases to predict the target gene of miRNAs[2]. The relative explanations were added in the discussion section on page 12, line 390-397.

Q4 Line 246, Page 8. The statistic method expressed in wrong words, the "Mann Whitney" should be "Mann Whitney U". Which statistic algorithm of authors to calculated ROC value is not shown in the manuscript? Another, "p" or "P" should be in a consistent manner.

Response:
The statistic algorithm used to calculate ROC value is regression. We have revised the error mentioned above in the manuscript.

Q5 In vitro analysis and validation should be performed to confirm the target genes.

Response:
We have gained the target gene of miR-375 predicted by four databases. In our subsequent study, we are going to validate the target genes in vitro and in vivo analysis and discuss the molecular mechanism of miR-375. The relative explanations were added in the section of discussion on page 12, line 396-397.

The references are as follows:
